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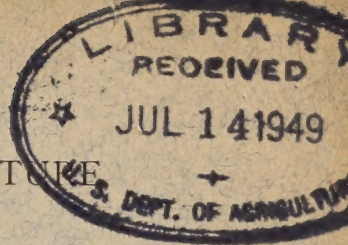
TECHNICAL SERIES, No. 14.

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U. S. DEPARTMENT OF AGRICULTURE

BUREAU OF ENTOMOLOGY.

L. O. HOWARD, Entomologist and Chief of Bureau.



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THE

BACTERIA OF THE APIARY,

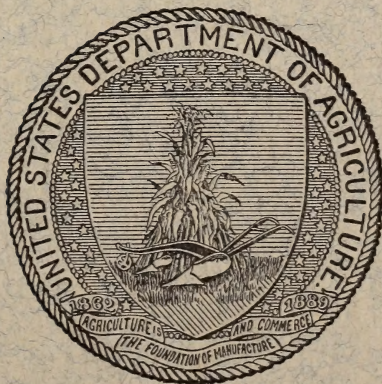
WITH SPECIAL REFERENCE TO  
BEE DISEASES.

BY

GERSHOM FRANKLIN WHITE, PH. D.,

*Expert in Animal Bacteriology, Biochemic Division, Bureau of Animal Industry.*

ISSUED NOVEMBER 6, 1906.



WASHINGTON:

GOVERNMENT PRINTING OFFICE.

1906.



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U. S. DEPARTMENT OF AGRICULTURE,  
BUREAU OF ENTOMOLOGY.

L. O. HOWARD, Entomologist and Chief of Bureau.

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## LETTER OF TRANSMITTAL.

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U. S. DEPARTMENT OF AGRICULTURE,  
BUREAU OF ENTOMOLOGY,  
*Washington, D. C., September 24, 1906.*

SIR: I have the honor to transmit the manuscript of a paper on the bacteria of the apiary, with special reference to bee diseases, by Dr. G. F. White, expert in animal bacteriology in the Biochemic Division of the Bureau of Animal Industry. This paper was prepared by Doctor White as a thesis in part fulfilment of the requirements for the degree of doctor of philosophy, at Cornell University, in June, 1905. The Bureau of Entomology considers itself fortunate in obtaining it for publication, since in this way a wider distribution can be made than would be possible were it published in a journal devoted exclusively to bacteriological investigations. It is hoped that the publication of these facts may help to clear up the confusion which now exists concerning the causes of the two most common diseases of the brood of bees. I recommend that the manuscript be published as Technical Series, No. 14, of this Bureau.

Doctor White wishes to acknowledge his indebtedness to Dr. Veranus A. Moore, professor of comparative pathology and bacteriology of Cornell University, under whose direction this work was done; to Dr. E. F. Phillips, acting in charge of apiculture, Bureau of Entomology, United States Department of Agriculture, for encouragement and assistance in the preparation of this manuscript; and to Messrs. Mortimer Stevens, Charles Stewart, N. D. West, and W. D. Wright, bee inspectors of the State of New York, for their interest in the work.

Respectfully,

L. O. HOWARD,  
*Entomologist and Chief of Bureau.*

HON. JAMES WILSON,  
*Secretary of Agriculture.*



## PREFACE.

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The spread of diseases of the brood of bees is to-day a great menace to the bee-keeping industry of the United States. It is therefore of great importance that all phases of these diseases should be investigated as thoroly as possible, and this paper, it is believed, will help in clearing up some disputed points in regard to the cause of the two most serious brood diseases.

Dr. G. F. White has offered this paper for publication as a bulletin in the Bureau of Entomology because in that way the statements herein contained may become more widely known than would be the case were it published in some journal devoted exclusively to bacteriological investigations. Obviously there are many points still unsettled, and it is hoped that some of these may be taken up for investigation in the near future, but the results so far obtained should by all means be made known to the persons practically engaged in bee keeping.

The necessity for the study of nonpathogenic bacteria found in the apiary may not be at first evident to the ordinary reader. When it is seen, however, that some of the investigators of bee diseases have apparently mistaken *Bacillus A* or some closely allied species for *Bacillus alvei* it will be evident that a study of nonpathogenic germs is necessary to a thoro investigation of the cause of these diseases and a full understanding of the confusion which has existed.

The names which should be used for the diseased conditions of brood was a matter which arose after this paper was offered for publication. It was desired that out of the chaos of names in use certain ones be chosen which would be distinctive and still clear to the bee keepers who are interested in work of this nature. Unfortunately, after a short investigation, Dr. W. R. Howard, of Fort Worth, Tex., gave the name "New York bee disease," or "black brood," to a disease which Cheshire and Cheyne described in 1885 as "foul brood." Since this is the disease in which *Bacillus alvei* is present, we can not drop the name "foul brood," and the word "European" is used to distinguish it from the other disease. The bee keepers of the United States have been taught that the type of brood disease characterized by ropiness of the dead brood is true foul brood,



but since *Bacillus alvei* is not found in this disease it obviously is not the same disease as that described by Cheyne. It would be well-nigh impossible, however, to change the name of this disease, and any effort in that direction would merely result in complicating laws now in force which control the infectious diseases of bees and would serve no good purpose. This disease is here designated "American foul brood." These names have been chosen only after consultation with some of the leading bee keepers of the United States, and these distinguishing terms were chosen by the majority of those consulted as indicating the place in which the diseases were first investigated in a thoroly scientific manner. Both diseases are found in Europe, as well as in America, so that the names indicate nothing concerning the geographical distribution of the maladies:

Strangely enough, certain writers for our American apicultural papers have seen fit to take exception to some of the statements made in this paper without having first found out the reasons for the decisions herein published. Apiculture will not be advanced to any appreciable extent by such eagerness to rush into print, especially when there is not a semblance of scientific investigation back of the criticism.

E. F. PHILLIPS,  
*Acting in Charge of Apiculture.*



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# THE BACTERIA OF THE APIARY WITH SPECIAL REFERENCE TO BEE DISEASES.

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## INTRODUCTION.

Since bacteriology is one of the youngest of the sciences, it is only natural that there should be many problems concerning which there is much confusion, and many others concerning which nothing is known. In a study of the saprophytic bacteria this is especially true; the exploration of this jungle of micro-organisms is scarcely begun. Comparatively few species have been studied and named, and a much less number can be identified. From studies that have been made one is led to believe that the species which might be classed under bacteria outnumber by far all the macroscopic plants known. Comparatively little is as yet known concerning the distribution of these minute organisms in nature, their needs for multiplication and growth, their power of endurance, their relations the one to the other, their relations to man and industries, and their relation to pathogenic species. Both from the standpoint of scientific interest and from the standpoint of practical economy these problems call for further investigation.

By far the greatest amount of work which has been done in the science of bacteriology has been prompted by the direct or indirect economic importance of the question. This is largely true of the present investigation, since honey bees suffer from a number of diseases, some of which are considered in Part II.

## TECHNIQUE.

### Obtaining Material for Study.

If necessary, bees may be conveniently shipped alive by mail in cages constructed for that purpose. Combs also may be sent by mail in small boxes. If combs, honey, pollen, or larvæ are desired, the hive must be entered. In case older adult bees are wanted it is not difficult to supply the needs from the entrance to the hive. To capture them one may stand at the entrance and catch the unwary toiler as she

comes in loaded with pollen and honey. After the victim alights on the entrance board, by the aid of a pair of forceps, before she disappears within, one can easily lodge her safely in a petri dish. It is, however, an advantage to study the young adult bees as well as the older ones, and if young ones are desired they may be taken from the combs or from the front of the hive, near the entrance.

#### Obtaining Cultures.

(a) *From combs.*—With sterile forceps small pieces of the comb are put directly into gelatin or agar for plates or incubated in bouillon for 24 hours and then plated. Growing in bouillon and plating on gelatin is usually preferable.

(b) *From pollen.*—The same technique is used as for combs, but the direct inoculation of gelatin tubes for plates is generally preferable.

(c) *From honey.*—With sterile loops honey is taken from uncapped and capped cells. The caps are removed with sterile forceps and the honey is plated directly on gelatin or agar. Bouillon tubes are inoculated also with varying quantities of the honey.

(d) *From larvæ.*—The larva is carefully removed to a sterile dish, and with sterile scissors the body is opened and the contents plated directly, or bouillon cultures are first made and later plated, if a growth appears.

(e) *From parts of the adult bee.*—In studying the adult bee, a small piece of blotting paper wet with chloroform is slipped under the cover of the petri dish in which the insects have been placed, and in a short time the bees are under the influence of the anesthetic. Then with sterile scissors a leg, a wing, the head, the thorax, or the abdomen, the intestine being removed, is placed in bouillon and, after 24 hours incubation, plated, preferably on gelatin.

When it is desired to make a study of the bacteria of the intestine, the intestinal tract is removed and studied as follows: The bee is flamed and held in sterile forceps. With another sterile pair of forceps the tip of the abdomen is seized and, by pulling gently, the tip and the entire intestine are easily removed. This can then be plated directly. If gelatin, which is preferable, is used, the intestine itself must not be left in the gelatin or the medium will become liquefied by the presence of the tissue. If one desires to obtain cultures of the anaërobe, which is quite common in the intestine, it is most easily obtained in pure culture by the use of the deep glucose agar (Liborius's method). Cover glass preparations made direct from the walls of the intestine or its contents give one some idea of the great number of bacteria frequently present.



### Differentiation and Identification of Bacteria.

These very low forms of plant life show a marked susceptibility to environmental conditions and those desirous of speculating on problems in evolution may find here food for thought and experimentation. On account of this susceptibility, various cultures which belong to the same species may possess slight variations in some one or more specific characters. Consequently one can not say that a species must possess certain definite characters and no others. It is convenient, then, to think of a species as more or less of a group of individuals whose characters approximate each other very closely.

In this paper are described a number of species each of which, in fact, represents a group, the individual cultures of which approximate each other so closely in character that the differences may be easily attributed to environmental conditions which are more or less recent.

Concerning the identification of species, the conditions have been well summed up by Chester. He says:

Probably nine-tenths of the forms of bacteria already described might as well be forgotten or be given a respectful burial. This will then leave comparatively few well-defined species to form the nuclei of groups in one or another of which we shall be able to place all new sufficiently described forms.

The variations which occur and the very incomplete descriptions which can be found make it impossible to identify many species even to a more or less restricted group. For these reasons some of the cultures are not identified or named, but letters are used for convenience in this paper to represent the specific part. Migula's classification has been used.

### The Cultures Which are Described.

Plate cultures were observed for some weeks, the different kinds of colonies which appeared being especially noted. Subcultures were then made in bouillon, and after 24 hours the subculture was replated. Subculturing and replating were then repeated. From this last plate the pure culture was made on agar for study. These were not studied culturally, as a rule, for some weeks, thus allowing time for the organism to eliminate any character due to recent environmental conditions (1).<sup>a</sup>

### Morphology, Staining Properties, and Oxygen Requirements, with Suggestions on Variations.

(a) *Size*.—The length and thickness of a micro-organism often varies so much with its environmental conditions that certain re-

<sup>a</sup> Numbers in parentheses refer to papers in the bibliography at the end of Part I or that at the end of Part II.

corded dimensions should always be accompanied by facts concerning the medium, age, and temperature of incubation. The measurements recorded in this paper were all taken of organisms in preparations made from a 24-hour agar culture stained with carbol-fuchsin. The involution forms are not reckoned in the results.

(b) *Spores*.—The presence of spores was determined in each case by staining the various cultures at different ages. A check was made on their presence by means of the thermal death point.

(c) *Flagella*.—Loeffler's method, as modified by Johnson and Mack, was used for staining the flagella (2).

(d) *Motility*.—Motility may be present in cultures when first isolated, but after artificial cultivation appear to be entirely lost. The reverse of this also may be noted. No cultures should be recorded as nonmotile until cultures on various media at different temperatures and of different ages shall have been studied. Hanging-drop preparations were made from cultures on agar and bouillon, both incubated and not incubated, and on gelatin.

(e) *Staining properties*.—Basic carbol-fuchsin was the stain used almost exclusively. In the use of Gram's staining method, carbolic gentian violet (5 per cent carbolic acid 20 parts, saturated alcoholic solution gentian violet 2 parts) was applied to a cover-glass preparation from a 24-hour culture on agar for 5 minutes, placed in Lugol's solution 2 minutes, and placed, without rinsing, in 95 per cent alcohol for 15 minutes, removed, washt in water, and allowed to dry.

(f) *Oxygen requirements*.—Determinations were made by observing whether a growth took place in the closed or open arm or both, of the fermentation tube containing glucose bouillon.

#### Media Employed and Suggestions as to the Description of Cultures.

(a) *Bouillon*.—All bouillon used was made from beef (meat 1 part, water 2 parts), to which infusion 1 per cent Witte's peptonum siccum and one-half per cent sodium chlorid were added. The reaction of the solution was then determined by titrating, and made +1.5 to phenolphthalein.

In describing a culture growing in bouillon as a medium, there is usually a more extended description given than in the case of sugar and sugar-free bouillons, since cultures in these media do not differ materially in gross appearance from those observed in the plain bouillon.

(b) *Sugar-free bouillon*.—This bouillon is made free from sugar by the use of *B. coli communis*, after which peptone and sodium chlorid (NaCl) were added as in bouillon.

(c) *Sugar bouillons*.—Five different sugars—glucose, lactose, saccharose, levulose, and maltose, as well as mannite—were used in the study. If a 1-per-cent solution of glucose in plain bouillon was fer-



mented with the production of gas, fermentation tubes were used for all the sugars and mannite. If no gas was formed in the glucose, the straight tubes were inoculated. The sugars and mannite were used in a 1-per-cent solution in sugar-free bouillon.

(d) *Reaction of media*.—The reaction of cultures is determined as it appears on the fifth day in the different media, unless otherwise stated. The medium in the open arm is used to determine the reaction in the fermentation tube. Beginning with a reaction of +1.5 to phenolphthalein, or slightly alkaline to litmus, the detection of an increase in acidity is not difficult. But inasmuch as the production of an alkali is very frequently small in degree, cultures are often in this paper recorded alkaline in reaction when probably the reaction has not changed.

(e) *Fermentation with the production of gas*.—Gas may be formed in such small quantities as not to be observed as such, but to be entirely absorbed by the medium. Whenever gas formation is mentioned as a character, visible gas is meant. The analysis of the gas was made in the usual manner by absorbing a portion with potassium hydrate (KOH) and testing the remainder with the flame. The amount absorbed by potassium hydrate (KOH) is referred to as carbon dioxid ( $\text{CO}_2$ ) and the remainder, if an explosion is obtained, as hydrogen (H). This is, naturally, only approximately correct. Since the gas formula may vary from day to day, too much value must not be given to the exact proportion. It is well to observe whether the proportion of hydrogen to carbon dioxid is greater or less than 1.

(f) *Agar*.—One per cent agar is used. The description of the growth on this medium is made from the appearance as seen on the surface of an agar slant. The description is usually very brief, since it has, as a rule, little differential value.

(g) *Acid agar*.—This medium is made acid by titrating to +3 to phenolphthalein. The absence or presence, as well as the degree of growth, is noted.

(h) *Serum*.—The serum used is taken from the horse, sterilized at  $55^\circ \text{C}$ . and congealed at  $80^\circ \text{C}$ . Deep inoculations are made, and the surface of slanted serum is also inoculated. The degree of growth is usually noted. Cultures are observed for 6 weeks to 2 months. The presence or absence of liquefaction is the chief character sought for. Since room temperature varies so greatly, the time at which liquefaction begins varies, and little differential value, therefore, can be given to the exact time of this phenomenon.

(i) *Potato*.—The composition of potato varies so markedly that a description of a culture on this medium may differ materially from that which is observed on another tube of the same medium. It is the aim to omit for the most part the observed variations due to the composition of the different potatoes.

(j) *Potato water*.—To potatoes sliced very thin is added an equal amount of water by weight and the mixture is then boiled. This is strained and distributed in straight and fermentation tubes. The reaction of the solution was made +1.5 to phenolphthalein. If any of the micro-organisms ferment glucose with the production of gas, fermentation tubes are inoculated to test the fermentation of starch; if not, straight tubes are inoculated.

(k) *Milk*.—If a micro-organism breaks up glucose with the formation of gas, a fermentation tube of milk is inoculated with the culture; if not, straight tubes are used. Separator milk is used. The coagulation of the casein with or without liquefaction is the chief character noted. Very little stress is laid upon the time element in the coagulation of the casein and the other phenomena which are to be observed in milk. Different samples of milk and the different environmental conditions are factors which vary the length of time at which the different phenomena appear.

(l) *Litmus milk*.—The reaction as shown by the litmus and the discharging of the color are the chief points observed.

(m) *Gelatin*.—The color, degree of growth, the presence or absence of liquefaction, and the form of liquefaction are the chief points observed. The cultures are kept under observation 2 months or longer and, as in serum, the time given at which liquefaction takes place is only approximate.

(n) *Indol*.—The cultures are allowed to grow in sugar-free peptonized bouillon for 3 to 5 days, and are tested with potassium nitrite ( $\text{KNO}_2$ ) and sulfuric acid ( $\text{H}_2\text{SO}_4$ ) after the ring method. Too much stress may be placed upon the ability of an organism to form indol. This character has been shown to be a somewhat transient one (3).

(o) *Reduction of nitrates to nitrites*.—Cultures are cultivated 7 days in a solution of 1 gram of Witte's peptonum siccum and one-fifth gram of sodium nitrate in 1,000 c. c. of tap water. To such a culture and to a control tube are added a mixture of naphthylamine and sulfanilic acid (naphthylamine, 1 part; distilled water, 1,000 parts; sulfanilic acid, one-half gram, dissolved in dilute acetic acid in the proportion of 1 part of acid to 16 parts of water). If nitrate is reduced to nitrite, a pink color develops. The control tube should remain clear, or slightly pink—owing to the absorption of a trace of nitrite from the atmosphere.

## PART I. BACTERIA OF THE NORMAL APIARY.

Before studying the cause of a disease it is necessary that we know what bacteria are normally present, so that later, in studying diseased conditions, a consideration of these nonpathogenic species may be eliminated. In view of this necessity a bacteriological study



of the hives, combs, honey, pollen, larvæ, and adult bees was begun, to determine the bacteria normally present. It was not hoped that all the species isolated could be easily identified, or that all would merit a careful description, but it was hoped that those species which seemed to be localized in any part of the apiary, or upon or within the bees, might be studied and described with sufficient care to guarantee their identification upon being isolated again. The chance of variation in morphology, pathogenesis, and cultural characters due to environmental conditions to which these micro-organisms were being subjected at the time, or to which they had been subjected before isolation or study, has been carefully borne in mind.

### BACTERIA FROM THE COMBS.

One might naturally suppose that very many species of bacteria would be present on combs, since these are exposed more or less to the contaminating influence of the air. The reverse, however, seems to be true. The number of different species isolated is comparatively small. Those which appear most often are described below. Some other species mentioned in this paper are found on combs, but inasmuch as they appear most frequently from other sources they are described there. One species of *Saccharomyces* from the comb, also, is described under the heading "*Saccharomyces* and fungi."

#### *Bacillus A.*

#### (*B. mesentericus*?)

*Occurrence.*—Found very frequently on combs, on scrapings from hives, and on the bodies of bees, both diseased and healthy.

*Gelatin colonies.*—Very young colonies show irregular edges, but very soon liquefaction takes place and the colony gives rise to a circular liquefied area, covered with a gray membrane, which later turns brown.

*Agar colonies.*—Superficial colonies present a very irregular margin consisting of outgrowths taking place in curves. Deep colonies show a filamentous growth having a moss-like appearance.

*Morphology.*—In the living condition the bacilli appear clear and often granular, arranged singly, in pairs, and in chains. The flagella are distributed over the body. The rods measure from  $3\mu$  to  $4\mu$  in length, and from  $0.9\mu$  to  $1.2\mu$  in thickness.

*Motility.*—The bacilli are only moderately motile.

*Spores.*—Spores are formed in the middle of the rod.

*Gram's stain.*—The bacilli take Gram's stain.

*Oxygen requirements.*—Aërobic and facultatively anaërobic.

*Bouillon.*—Luxuriant growth in 24 hours, with cloudiness of medium; a gray flocculent membrane is present. Later, the membrane sinks and the medium clears, leaving a heavy, white, flocculent sediment, with a growth of the organisms adhering to the glass at the surface of the medium. Reaction alkaline.

*Glucose.*—Luxuriant growth takes place in the bulb, with a moderate, flocculent growth in closed arm. The gradual settling of the organisms causes a

heavy white sediment to form in the bend of the tube. The reaction is at first slightly acid, but subsequently becomes alkaline. No gas is formed.

*Lactose*.—Reaction alkaline.

*Saccharose*.—Reaction alkaline.

*Levulose*.—Reaction acid.

*Maltose*.—Reaction acid.

*Mannite*.—Reaction alkaline.

*Potato water*.—Reaction alkaline.

*Agar slant*.—A luxuriant growth takes place on this medium. The growth gradually increases to a moist, glistening one, being then friable and of a grayish brown color.

*Serum*.—A luxuriant, brownish, glistening, friable growth spreads over the entire surface. No liquefaction is observed.

*Potato*.—An abundant fleshy growth of a brown color spreads over the entire surface. The water supports a heavy growth. The potato is slightly discolored.

*Milk*.—Precipitation takes place rapidly, followed by a gradual digestion of the casein, the medium changing from the top downward to a translucent liquid, becoming at last semi-transparent and viscid.

*Litmus milk*.—Precipitation of the casein takes place usually within 24 hours, followed by a gradual peptonization. Reduction of the litmus occurs rapidly, leaving the medium slightly brown; later the blue color will return on exposing the milk to the air by shaking. Reaction alkaline.

*Gelatin*.—An abundant growth takes place with rapid, infundibuliform liquefaction. A heavy, white, friable membrane is formed on the surface of the liquefied medium. A flocculent sediment lies at the bottom of the clear liquefied portion.

*Acid agar*.—Growth takes place.

*Indol*.—None has been observed.

*Nitrate*.—Reduction to nitrite is positive.

### ***Bacterium acidiformans*. (Sternberg, 1892.)**

*Occurrence*.—Isolated from the scraping of propolis and wax from the hives and frames of healthy colonies.

*Gelatin colonies*.—The superficial colonies are friable, convex, opaque, and white with even border; when magnified they are finely granular, sometimes radiately marked. They are from 1 to 4 millimeters in diameter. The deep colonies are spherical or oblong and entire.

*Morphology*.—When taken from an agar slant 24 hours old, the rods are short, with rounded ends, singly and in pairs. Length about  $1.6\mu$ , thickness  $0.8\mu$ . They stain uniformly with carbol-fuchsin. Flagella are apparently absent.

*Motility*.—No motility has been observed in any medium.

*Spores*.—Spores are apparently absent.

*Gram's stain*.—The bacteria are decolorized by Gram's method.

*Oxygen requirements*.—Facultatively anaërobic.

*Bouillon*.—The medium becomes slightly clouded with a feeble ring of growth on the glass at the surface of the liquid. A moderate amount of white friable sediment is formed. Reaction alkaline.

*Glucose*.—Uniformly and slightly clouded. No gas is formed. Reaction acid.

*Lactose*.—Reaction acid.

*Saccharose*.—Reaction alkaline.

*Levulose*.—Reaction acid.



*Maltose*.—Reaction acid.

*Mannite*.—Reaction acid.

*Potato water*.—Reaction acid.

*Agar slant*.—A moderate, gray, glistening growth, confined to the area inoculated with the loop, is formed on the inclined surface.

*Serum*.—A feeble gray growth is formed only on the inoculated surface. No liquefaction takes place.

*Potato*.—A gray growth covers the inoculated surface.

*Milk*.—Heat causes a ready coagulation of the casein. Reaction acid.

*Litmus milk*.—Coagulation of casein occurs promptly on boiling a culture 2 weeks old. Reaction acid.

*Gelatin*.—Growth of spherical colonies appears along the line of inoculation, the surface growth being grayish and spreading slowly. No liquefaction takes place.

*Acid agar*.—Growth takes place.

*Indol*.—A trace was observed.

*Nitrate*.—No reduction to nitrite could be observed.

### BACTERIA FROM POLLEN.

As in the case of the examination of the combs, the number of species of bacteria found in pollen is comparatively small. The following are often found to be present. Other species have been isolated, but their distribution in the pollen is not at all constant.

#### *Bacillus B.*

*Occurrence*.—Found frequently in pollen and in the intestine of healthy honey bees.

*Gelatin colonies*.—The colonies are egg-yellow with even border. Liquefaction takes place slowly. Surface colonies are about 1.5 millimeters in diameter, have coarsely granular center, finely granular margin, and clear and sharply defined border. A peculiar toruloid growth is often observed.

*Morphology*.—The organisms are short rods with rounded ends, which stain uniformly with carbol-fuchsin, and are  $1\mu$  to  $2\mu$  in length. Few short involution forms occur.

*Motility*.—The bacilli are actively motile in young cultures.

*Spores*.—No spores have been observed.

*Gram's stain*.—The bacilli are decolorized by Gram's stain.

*Oxygen requirements*.—Facultatively anaërobic.

*Bouillon*.—This medium becomes uniformly clouded, frequently with a scanty, friable membrane. Sometimes the organisms settle, clearing the medium and forming a viscid sediment. A growth of the culture adheres to the glass at the surface of the liquid. This, together with the membrane, is of a light egg-yellow color, which deepens somewhat with age. Reaction alkaline.

*Glucose*.—At first both arms of the fermentation tube are clouded slightly, and the cloudiness later increases. Sometimes a stronger growth occurs in the closed arm than in the open one. Reaction is at first acid, but slowly changes to alkaline.

*Lactose*.—Reaction alkaline.

*Saccharose*.—Reaction alkaline.

*Levulose*.—Reaction alkaline.

*Maltose*.—Reaction slightly acid.

*Mannite*.—Reaction slightly acid, later alkaline.

*Agar slant*.—A moderate, slightly yellow, nonviscid glistening growth appears along the inoculated surface. This growth gradually spreads and deepens in color to an egg-yellow.

*Potato*.—A moderate, egg-yellow, nonviscid, glistening growth spreads over the entire surface. The potato is slightly discolored.

*Milk*.—The milk is covered by a yellow growth of the culture, resembling cream. Coagulation takes place on boiling.

*Litmus milk*.—Reaction alkaline.

*Gelatin*.—Growth takes place along the line of inoculation. Deep in the medium the colonies are white and spherical; the surface growth is yellow. After a few days liquefaction begins, and at the end of 2 weeks one-half the tube is liquefied. The liquefaction is infundibuliform. Liquefied gelatin is surmounted by a friable, egg-yellow pellicle. The growth in the liquefied portion is flocculent, which, on settling, forms a yellow sediment at the apex.

*Indol*.—None could be observed.

*Nitrates*.—No reduction to nitrites occurs.

### BACTERIA IN HONEY AND NORMAL LARVÆ.

Comb honey from a large number of sources has been examined and found to be quite uniformly sterile. The healthy larvæ likewise are usually sterile.

### BACTERIA UPON THE ADULT BEES.

On the external part of the bee we again find only a few different species. *Bacillus A*, described as found upon the combs, is frequently isolated from the bee. Other species which are found frequently are described below.

#### *Bacterium cyaneus* (*Micrococcus cyaneus*).

*Occurrence*.—Isolated from the body of a healthy honey bee and from pollen.

*Gelatin colonies*.—The colonies are lemon-yellow, with entire border, growth taking place readily on this medium. The superficial colonies, having well-defined border, are finely granular, and liquefy the medium within 3 to 6 days.

*Morphology*.—Short oval rods  $0.8\mu$  to  $1.7\mu$  in length,  $0.7\mu$  to  $0.8\mu$  in thickness. Short involution forms are present. The rods occur singly, paired, and in clumps. No flagella have been demonstrated.

*Motility*.—No motion has been demonstrated.

*Spores*.—No spores have been demonstrated.

*Gram's stain*.—The bacterium takes Gram's stain.

*Oxygen requirements*.—Aërobic.

*Bouillon*.—At first a slight cloudiness appears, the medium becoming turbid in old cultures. A heavy yellowish-white, slightly viscid ring forms on the tube at the surface of the medium. The sediment, and sometimes the medium, show marked viscosity. Reaction alkaline.

*Glucose*.—The growth of the culture is confined entirely to the open bulb, in which the medium becomes turbid. No gas is formed. Reaction alkaline.

*Lactose*.—Reaction alkaline.

*Saccharose*.—Reaction alkaline.

*Levulose*.—Reaction alkaline.



*Maltose*.—Reaction alkaline.

*Mannite*.—Reaction alkaline.

*Potato water*.—Reaction alkaline.

*Agar slant*.—On the surface of the agar there takes place an abundant growth, which is confined to the surface inoculated with the loop. The culture is fleshy, nonviscid, and lemon-yellow. It produces a soluble pigment that diffuses thru the agar, giving it a dark-pink color.

*Serum*.—Luxuriant growth takes place, accompanied by liquefaction.

*Potato*.—A lemon-yellow, fleshy, glistening growth spreads over the inclined surface of the potato.

*Milk*.—Precipitation followed by slow liquefaction of the casein occurs; later the medium becomes alkaline and very viscid.

*Litmus milk*.—The litmus is discharged and the casein is liquefied. Reaction alkaline.

*Gelatin*.—Infundibuliform liquefaction soon begins, which is followed by stratiform liquefaction. The liquefied gelatin is turbid and viscid.

*Acid agar*.—On this medium a moderate lemon-yellow growth is observed.

*Indol*.—None could be observed.

*Nitrates*.—No reduction of nitrates could be observed.

### Micrococcus C.

*Occurrence*.—Isolated from the body of a healthy honey bee.

*Gelatin colonies*.—The surface colonies are round and slightly yellow. Liquefaction begins in from 2 to 4 days. The magnified colonies are finely granular, with sharply defined, entire border.

*Morphology*.—Cocci, about  $0.8\mu$  in diameter, occur in pairs and in small clusters.

*Motility*.—Nonmotile.

*Spores*.—Spores are apparently absent.

*Gram's stain*.—The coccus takes the Gram's stain.

*Oxygen requirements*.—Aërobic.

*Bouillon*.—This medium becomes uniformly clouded in 24 hours after inoculation, growth increases, and friable sediment forms. The liquid clears somewhat on standing. Reaction at first slightly acid; later returns to neutral.

*Glucose*.—The medium in the bulb becomes cloudy, while that in the closed arm remains clear. White friable sediment forms in bend of tube. Reaction acid. No gas is formed.

*Lactose*.—Reaction slowly becomes acid.

*Saccharose*.—Reaction acid.

*Levulose*.—Reaction acid.

*Maltose*.—Reaction acid.

*Mannite*.—Reaction acid.

*Potato water*.—Reaction acid.

*Agar slant*.—A grayish white, fleshy, nonviscid, glistening growth takes place along the inoculated surface. It does not spread, and retains a distinct boundary.

*Serum*.—A spreading growth takes place, accompanied by liquefaction.

*Potato*.—A gray, fleshy, glistening, nonviscid growth forms over the entire cut surface of the potato. The potato is slightly discolored.

*Milk*.—This medium becomes firmly coagulated and later the casein liquifies with the formation of a milky serum.

*Litmus milk*.—In this medium coagulation takes place, accompanied by reduction of the litmus. Reaction slightly acid.

*Gelatin*.—After a day or two infundibuliform liquefaction occurs, being followed by stratiform liquefaction; the liquefied gelatin is turbid. Growth below this portion is in the form of small spherical colonies.

*Acid agar*.—A white, fleshy, nonviscid growth is observed.

*Indol*.—A trace was observed.

*Nitrates*.—Reduced to nitrites.

### BACTERIA OF THE INTESTINE OF THE HEALTHY HONEY BEE.

A great many investigations have been made in recent years on the bacteria found present in the intestines of vertebrates (4, 5, 6, 7, 8, 9), and striking similarities are noticed in the species found in many of them. In this investigation the intestinal contents of about 150 bees, mostly from one apiary, have been studied more or less thoroly. Several species which are found to be constant in many of the vertebrates are found in the intestine of the honey bee. Since the temperature of the bee approximates much of the time, especially when in the hive, that of the warm-blooded animals, many of the same species of bacteria inhabit the intestine of this insect as are found thriving in the same locality in man and other animals. A stained cover-glass preparation made directly from a healthy adult field bee reveals, almost without exception, a multitude of bacteria.

In a study of the bacterial flora stress has been placed upon the different species which were found to be more or less constant, rather than upon the actual number of bacteria or species in any quantity of material from a single bee. From the observations which have been made, it appears that the number of species in any individual is comparatively small, but the number of bacteria is in many cases very large. Sometimes, however, the plates show very few colonies, while cover-glass preparations show a very large number of bacteria. These organisms are probably the anaërobe, which is quite constant, as shown by cultures made direct from the intestine into glucose agar (Liborius's method).

When a loopful of the material from the intestine was used for the inoculation, the following data give the approximate findings:

Bee No. 1, 300 to 400 yellow colonies, probably alike.

Bee No. 2, a few colonies of fungi only.

Bee No. 3, 500 colonies, mostly yeast.

Bee No. 4, 100 or more colon-like colonies.

Bee No. 5, 2,000 or more, mostly yellow.

Bee No. 6, 20 or more colonies, mostly yeasts.

Bee No. 8, 400 or more yellow colonies.

Bee No. 9, 30 yeasts with a few fungi.

Bee No. 10, 50 yeast colonies with a few fungi.

Bee No. 11, no growth.

Bee No. 12, 300 colonies, slightly yellow.



Bee No. 13, 2,000 or more gray colonies.

Bee No. 14, yeast colonies and a few colonies of bacteria showing ground-glass appearance.

Bee No. 15, 2,000 or more colon-like colonies (*B. cloacæ*).

The following are the species which have been found to be most constant. The reader is referred also to the description of the yeast plant found very frequently in the intestine of the normal honey bee, described under "*Saccharomyces* and fungi."

#### **Bacterium D.**

*Occurrence*.—Frequent in the intestine of the healthy honey bee.

*Agar colony*.—Deep colonies when magnified are coarsely granular, showing a dark brown center, with a thin and ill-defined border.

*Morphology*.—A preparation made from a young culture taken from a glucose fermentation tube shows rods with rounded ends, occurring singly and in pairs, staining easily and uniformly with carbol-fuchsin, and measuring  $0.7\mu$  to  $1.5\mu$  in length and  $0.5\mu$  to  $0.7\mu$  in thickness.

*Motility*.—No motility could be observed.

*Spores*.—No spores could be demonstrated in young cultures. In old cultures their presence is questionable.

*Oxygen requirements*.—Strictly anaërobic.

*Bouillon*.—In straight tubes no growth occurs.

*Glucose*.—A moderate cloudiness can be seen in the closed arm, while the open bulb remains clear. No gas is produced. Reaction about neutral.

*Glucose agar* (Liborius's method).—Growth is rather slow. After 3 days a moderate growth may be observed; later, if cultures have recently been isolated from the bee's intestine, the growth imparts to the medium a diffused haziness or cloudiness. After many generations the culture loses this property.

*Glucose gelatin* (Liborius's method).—Very slow growth occurs in the depth of the medium. No liquefaction takes place.

#### **Bacillus cloacæ.**

*Occurrence*.—Found in the intestine of a large number of healthy honey bees.

*Gelatin colonies*.—Superficial colonies are thin and blue to gray in color; deep colonies, brown, regular, granular, and spherical to lenticular.

*Agar colonies*.—Superficial colonies are partially opaque, brown, finely granular, with well-defined margin; deep colonies are regular, spherical, or lenticular, with well-defined margin.

*Morphology*.—The rods from 24-hour agar cultures have rounded ends, varying in length from  $1\mu$  to  $2\mu$  and in width from  $0.7\mu$  to  $0.9\mu$ . They are usually found singly or in pairs. Involution forms are not uncommon. With carbol-fuchsin they stain uniformly. This species possesses a few peritrichic flagella.

*Motility*.—Active motility is observed in young cultures.

*Spores*.—No spores are formed.

*Gram's stain*.—The bacillus does not take Gram's stain.

*Oxygen requirements*.—Facultatively anaërobic.

*Bouillon*.—A uniform cloudiness appears in 24 hours. Growth continues until the medium becomes heavily clouded, followed by a gradual settling of many of the organisms, forming a viscid grayish-white sediment. A gray friable membrane, which adheres to the sides of the tube at the surface of the medium, is sometimes produced. Upon agitation this membrane breaks up and sinks to the

bottom, leaving a gray ring of the growth adhering to the glass. Reaction alkaline.

*Glucose*.—The medium in the bulb becomes turbid, while that in the closed arm is uniformly cloudy. A heavy grayish-white sediment is formed. The reaction is at first slightly acid, but in a few days becomes alkaline. Abundant and rapid gas formation takes place, filling usually from one-half to nine-tenths of the closed arm. The ratio of hydrogen to carbon dioxid is approximately 1 to 2; that is, the ratio of hydrogen to carbon dioxid is less than 1.

*Lactose*.—In this medium gas formation takes place more slowly than in glucose. At the end of 8 days one-fourth of the closed arm is filled with gas. The ratio of hydrogen to carbon dioxid is greater than 1. Reaction acid.

*Saccharose*.—Gas is formed abundantly and rapidly; more than one-half of the tube is usually filled with gas. The ratio of hydrogen to carbon dioxid is less than 1. Reaction alkaline.

*Levulose*.—A rapid fermentation takes place; more than one-half of the closed arm is filled with gas. The ratio of hydrogen to carbon dioxid is approximately 1 to 5; that is, less than 1. A slight formation of acid takes place at first, but the reaction rapidly becomes alkaline.

*Maltose*.—Formation of gas takes place with the result that at the end of 5 days approximately one-half of the tube is filled. The ratio of hydrogen to carbon dioxid will approximate that of 1 to 1. Reaction acid.

*Mannite*.—Gas is formed rapidly and abundantly; at the end of 5 days the closed arm is usually much more than half filled with the gas. The reaction is at first slightly acid, but soon becomes alkaline. The ratio of hydrogen to carbon dioxid is approximately 1 to 2; that is, less than 1.

*Potato water*.—Gas forms rapidly and fills half the closed arm. The ratio of hydrogen to carbon dioxid is as 1 to 2; that is, less than 1.

*Agar slant*.—A moderate, grayish-white, glistening, friable growth appears along the line of inoculation, which usually spreads to the sides of the tube.

*Serum*.—Moderate gray growth appears, which is confined quite closely to the line of inoculation. Liquefaction takes place slowly after 3 weeks.

*Potato*.—A moderate amount of gray fleshy growth covers the slope. The potato is slightly discolored.

*Milk*.—Coagulation takes place after 4 days' growth. Gas is formed.

*Litmus milk*.—A marked production of acid takes place, followed by firm coagulation.

*Gelatin*.—A heavy white growth takes place along the line of inoculation; the surface growth is flat, bluish-white, and spreads with an uneven margin. Slow infundibuliform liquefaction takes place after 2 weeks.

*Acid agar*.—A growth takes place.

*Indol*.—A trace is sometimes produced.

*Nitrates*.—Reduction to nitrites is positive.

### **B. coli communis.**

*Occurrence*.—Found in the intestine of healthy honey bees.

*Gelatin colonies*.—The superficial colonies are blue, lobate-lobulate, and slightly spreading; when magnified they are brownish yellow in the center and more transparent toward the margin; the deep colonies are spherical to lenticular and brownish yellow, with well-defined borders.

*Morphology*.—The short rods with rounded ends measure  $1.5\mu$  to  $2\mu$  in length and  $0.7\mu$  to  $0.8\mu$  in thickness. They occur singly or in pairs, stain uniformly, and are motile by means of a few peritrichic flagella.



*Motility*.—The bacilli are actively motile from some cultures.

*Spores*.—No spores are formed.

*Gram's stain*.—The bacillus is decolorized by Gram's method.

*Oxygen requirements*.—It is a facultative anaërobe.

*Bouillon*.—The medium becomes uniformly clouded in 24 hours, with a slight acid reaction; the medium later becomes alkaline, with a gray and friable sediment. A feeble pellicle is formed and a growth of the organism often adheres to the glass at the surface of the liquid.

*Glucose*.—Both branches of the fermentation tube become clouded. The sugar splits by fermentation into gas and acid, one-half or more of the closed arm being filled. The ratio of hydrogen to carbon dioxid is 2 to 1.

*Lactose*.—Gas fills one-fourth of the closed tube. Reaction acid.

*Saccharose*.—Gas fills one-sixth of the closed tube. Reaction acid.

*Levulose*.—Gas fills one-half of the closed tube. The value of hydrogen to carbon dioxid is 2 to 1. Reaction acid.

*Maltose*.—One-sixth of the closed arm is filled with gas. Reaction acid.

*Mannite*.—One-half of the closed tube is filled with gas. Reaction acid.

*Potato water*.—Reaction acid.

*Agar slant*.—A moderate, gray, nonviscid, spreading growth takes place on the surface of the inclined agar.

*Serum*.—A gray, glistening, nonspreading growth is observed on the inclined serum. No liquefaction takes place.

*Potato*.—A moderate, fleshy, glistening growth spreads over the inoculated surface. Potato slightly discolored.

*Milk*.—Coagulation of the casein takes place in about 4 days. A small quantity of gas is produced.

*Litmus milk*.—Coagulation occurs. Reaction strongly acid.

*Gelatin*.—A moderate growth occurs along the line of inoculation; the growth is spreading with an irregular margin on the surface. No liquefaction occurs.

*Acid agar*.—A moderate grayish growth occurs on surface.

*Indol*.—A trace was obtained in some cultures.

*Nitrates*.—Reduced to nitrites.

### B. *cholerae suis*.

*Occurrence*.—Isolated from the intestine of healthy honey bees.

*Gelatin colonies*.—Colonies are translucent by transmitted light; bluish to gray by reflected, the border being uneven and well defined. When the colonies are magnified they appear brownish and finely granular.

*Morphology*.—The rods are short, with rounded ends, occurring singly and in pairs, and staining uniformly with carbol-fuchsin, 1 to  $2.8\mu$  in length, and  $0.6\mu$  to  $0.8\mu$  in thickness. A few peritrichic flagella are present.

*Motility*.—Usually only a few are motile at a time in the field, and these present a rapid whirling motion.

*Spores*.—No spores are formed.

*Gram's stain*.—The bacteria are decolorized by Gram's stain.

*Oxygen requirements*.—Facultatively anaërobic.

*Bouillon*.—A uniform, moderate cloudiness arises in this medium in 24 hours; later a grayish-white membrane is formed which, upon shaking the tube, sinks to the bottom, forming a gray sediment. The reaction is at first slightly acid, but later becomes alkaline.

*Glucose*.—The medium becomes clouded in both arms of the fermentation tube, with the production of a small amount of gas. Reaction acid.

*Lactose*.—Growth takes place in both arms of the tube, but the sugar is not split into either acid or gas.

*Saccharose*.—Growth occurs in both arms of the tube, neither acid nor gas being formed.

*Levulose*.—Growth takes place in both arms with the production of gas and acid; one-third of the closed arm is filled. The ratio of hydrogen to carbon dioxid is about 3 to 1—that is, greater than 1.

*Maltose*.—The medium in both arms of the tube becomes clouded. Fermentation results in the production of gas sufficient to fill about one-fifth of the tube. Only a small portion of the gas is absorbed by sodium hydroxid, leaving behind an explosive gas.

*Mannite*.—The medium in both branches of the tube becomes clouded; gas is not formed. Reaction alkaline.

*Potato water*.—About one-fifth of the closed arm is filled with gas. Reaction acid.

*Agar slant*.—A moderate, grayish-white, glistening, nonspreading growth is formed along the surface inoculated with the loop.

*Serum*.—A moderate, gray, glistening, nonspreading growth takes place on the inclined surface. No liquefaction occurs.

*Potato*.—A feeble, grayish growth is observed. The potato becomes slightly discolored.

*Milk*.—No coagulation occurs, and no gas is produced. Reaction alkaline.

*Litmus milk*.—The medium slowly becomes more and more alkaline.

*Gelatin*.—A moderate, white growth takes place along the line of inoculation. On the surface it spreads with irregular margin. No liquefaction occurs.

*Acid agar*.—A moderate growth appears.

*Indol*.—Indol is produced.

*Nitrates*.—Reduction to nitrites (?).

### Bacillus E.

*Occurrence*.—Isolated from the intestine of healthy honey bees.

*Gelatin colonies*.—The colonies are lemon-yellow. Surface colonies are convex, smooth, with entire margin; when magnified they are finely granular. Deep colonies, when magnified, are lenticular, finely granular, and may appear dark green. Liquefaction takes place slowly.

*Morphology*.—The rods are short, with rounded ends, and usually occur singly. The bacilli are  $1.5\mu$  to  $2\mu$  in length and  $0.7\mu$  in thickness. This species possesses a few peritrichic flagella.

*Motility*.—The bacteria are actively motile.

*Spores*.—No spores are present.

*Gram's stain*.—They stain with Gram's stain.

*Oxygen requirements*.—Aërobic.

*Bouillon*.—The medium becomes uniformly clouded in 24 hours. Later a tough, yellowish-white membrane is formed, which sinks upon shaking. The medium is very viscid in old cultures. Reaction alkaline.

*Glucose*.—Growth is confined to the open bulb. No gas formation occurs. Reaction slightly acid.

*Lactose*.—There is a marked mucous-like appearance in the medium. Reaction alkaline.

*Saccharose*.—Reaction acid.

*Levulose*.—Reaction alkaline.

*Maltose*.—Reaction alkaline.

*Mannite*.—Reaction slightly acid.



*Potato water*.—Reaction alkaline.

*Agar slant*.—A moderate, yellowish-gray, nonviscid growth takes place on the surface.

*Serum*.—A strong growth takes place and the medium is liquefied.

*Potato*.—A yellowish-gray, nonviscid growth is observed over the entire inclined surface.

*Milk*.—Precipitation of casein takes place with very slight digestion (?).

*Litmus milk*.—Precipitation of the casein occurs. Reaction alkaline.

*Gelatin*.—A white growth forms along the line of inoculation, which becomes slowly liquefied from above.

*Acid agar*.—A moderate, slightly yellow growth is observed.

*Indol*.—None demonstrated.

*Nitrates*.—No reduction to nitrites occurs.

### **Bacillus subgastricus.**

*Occurrence*.—Isolated from the intestine of a healthy honey bee.

*Gelatin colony*.—The colon-like, superficial colonies are thin, blue, spreading, and lobate-lobulate. When magnified they are finely granular, with brown center. Deep colonies are spherical and yellow.

*Morphology*.—Short rods, singly and in pairs, are from  $1.5\mu$  to  $2.5\mu$  long and from  $0.6\mu$  to  $0.8\mu$  thick. They stain uniformly with carbol-fuchsin.

*Motility*.—Marked whirling motion from gelatin cultures.

*Spores*.—No spores could be demonstrated.

*Gram's stain*.—The bacilli are decolorized with Gram's stain.

*Oxygen requirements*.—Facultatively anaërobic.

*Bouillon*.—This medium becomes clouded in 24 hours. A slight band of growth is formed on the glass at the surface of the liquid. Later a feeble pellicle is sometimes formed. Reaction at first slightly acid, later becomes alkaline.

*Glucose*.—The medium in both branches of the tube becomes clouded. Gas is readily formed until about one-fourth of the closed branch is filled. The ratio of hydrogen to carbon dioxid is 2 to 1—that is, greater than 1. Reaction strongly acid.

*Lactose*.—Gas formation occurs. About one-sixth of the tube is filled with gas, part of which is absorbed by sodium hydroxid and another part is explosive. Reaction acid.

*Saccharose*.—This sugar is fermented to the point of formation of acid, but no gas is formed.

*Levulose*.—This sugar splits in the process of fermentation to form acid and gas, the gas filling about one-sixth of the tube. A portion of the gas is absorbed by sodium hydroxid, the remainder being explosive.

*Maltose*.—Fermentation takes place with the formation of acid. No gas is produced.

*Mannite*.—One-fifth of the closed arm is filled with gas. A portion of the gas is absorbed by sodium hydroxid and a portion is explosive. Reaction acid.

*Potato water*.—Reaction alkaline.

*Agar slant*.—A moderate, translucent, gray, nonviscid and glistening growth spreads slowly from the surface inoculated with the loop.

*Serum*.—A moderate, glistening growth appears along the surface inoculated. No liquefaction occurs.

*Potato*.—A grayish growth takes place on the sloped surface.

*Milk*.—Firm coagulation of the milk takes place with the formation of a small amount of clear serum. A small amount of gas is produced.

*Litmus milk*.—Reaction strongly acid. Coagulation occurs in about six days.

*Gelatin*.—White, spherical colonies appear along the line of inoculation. The surface growth is grayish blue and spreading, with irregular margin. Slow liquefaction takes place, beginning usually in 2 weeks.

*Acid agar*.—A growth takes place.

*Indol*.—None could be demonstrated.

*Nitrates*.—No reduction to nitrites occurs.

### **Bacterium mycoides.**

*Occurrence*.—Isolated from the intestine of a healthy honey bee.

*Gelatin colonies*.—A rapid growth of root-like colonies appears in 24 hours. In macroscopic appearance it somewhat resembles cotton fibers; when magnified these appear thick and somewhat felted in the center, while toward the margin they are beautifully filamentous. After a day or two the gelatin begins to liquefy.

*Morphology*.—The rods are large, scarcely rounded at the ends, and frequently in chains. They measure from  $2.5\mu$  to  $5.5\mu$  long and  $1.5\mu$  thick. No flagella have been demonstrated.

*Motility*.—No motility could be demonstrated.

*Spores*.—Spores are present.

*Gram's stain*.—The bacteria are not decolorized by Gram's stain.

*Oxygen requirements*.—Facultatively anaërobic.

*Bouillon*.—A decided fleecy growth with heavy, cotton-like sediment occurs.

*Glucose*.—No gas is formed. Reaction acid.

*Lactose*.—Reaction acid.

*Saccharose*.—Reaction acid.

*Levulose*.—Reaction acid.

*Maltose*.—Reaction acid.

*Mannite*.—Reaction acid.

*Potato water*.—Reaction alkaline.

*Agar slant*.—A luxuriant growth that appears root-like takes place on this medium. This growth tends to extend into the agar, which causes it to adhere to the medium.

*Serum*.—A luxuriant growth is formed, accompanied by liquefaction.

*Potato*.—A thick, gray, moist growth is found, the potato not being discolored.

*Milk*.—Coagulation occurs promptly, with formation of a clear serum.

*Litmus milk*.—The color is discharged in 48 hours.

*Gelatin*.—Hair-like outgrowths occur along the line of inoculation. Liquefaction begins at the surface and proceeds along the needle tract. In a few days the entire medium is liquefied.

*Indol*.—No indol is produced.

*Nitrates*.—Reduction to nitrites is positive.

### **Pseudomonas fluorescens liquefaciens.**

*Occurrence*.—Isolated from the intestine of the healthy honey bee.

*Gelatin colonies*.—Before liquefaction, the superficial colonies, when magnified, are finely granular, with regular margin; deep colonies are spherical, brown, with regular margin. Liquefaction takes place rapidly. The surface of liquefied gelatin is covered by a friable membrane. Later the liquefied gelatin takes on a green fluorescence.

*Morphology*.—The bacteria are short rods, varying from  $1\mu$  to  $2\mu$  in length and from  $0.5\mu$  to  $0.7\mu$  in thickness. They stain uniformly with carbol-fuchsin and are motile by means of one or more polar flagella.



*Spores*.—No spores could be demonstrated.

*Gram's stain*.—The bacteria do not take Gram's stain.

*Oxygen requirements*.—Aërobic

*Temperature requirements*.—Culture must be grown at room temperature.

*Bouillon*.—The medium becomes clouded in 48 hours, forming a moderately tough pellicle. A greenish-yellow fluorescence begins at the surface, which gradually increases until the entire medium takes on that appearance. Reaction alkaline.

*Glucose*.—A cloudiness is formed in the open arm, but the closed arm is clear. Reaction alkaline.

*Lactose*.—Reaction alkaline.

*Saccharose*.—Reaction alkaline.

*Levulose*.—Reaction alkaline.

*Maltose*.—Reaction alkaline.

*Mannite*.—Reaction alkaline.

*Agar slant*.—At first a gray friable growth is formed confined to the surface inoculated, which later takes on a brown hue. Greenish-yellow fluorescence is observable in the medium.

*Serum*.—A slow liquefaction occurs.

*Potato*.—Very scanty growth occurs with slight discoloration.

*Milk*.—Rapid liquefaction of the casein takes place.

*Litmus milk*.—Rapid liquefaction of the casein takes place. Reaction alkaline.

*Gelatin*.—Infundibuliform liquefaction takes place rapidly.

*Acid agar*.—No growth occurs.

*Indol*.—No indol observed.

*Nitrates*.—No reduction to nitrites occurs.

## SACCHAROMYCES AND FUNGI.

The first yeast plant described below is of very frequent occurrence in the intestine of the normal bee. *Saccharomyces roseus* can be isolated from the comb. A large number of common fungi were found in the flora of the intestines and in cultures from the pollen and combs.

In addition to the above the third *Saccharomyces* here described was found in two samples of brood apparently diseased, which could not be diagnosed as any disease commonly known.

### *Saccharomyces F.*

*Occurrence*.—Very common in the intestine of healthy honey bees.

*Gelatin colonies*.—Colonies form slowly; the superficial colonies are white, glistening, convex, capitate, and about 1 to 2 millimeters in diameter. When magnified they are finely granular, brownish yellow, with entire margin. Deep colonies are finely granular, with uniform margin, spherical to lenticular, and brownish green.

*Morphology*.—The cells are oval and on agar in 24 hours attain their full size of  $4.5\mu$  in length and  $3.5\mu$  in thickness. They stain uniformly with carbol fuchsin.

*Motility*.—The yeast is not motile.

*Gram's stain*.—The cells take the Gram's stain.

*Oxygen requirements*.—Aërobic

*Bouillon*.—This medium remains clear, with the formation of a friable white sediment. Reaction neutral.

*Glucose*.—The closed arm remains clear. No gas is formed. Reaction acid.

*Lactose*.—Reaction neutral.

*Saccharose*.—Reaction neutral.

*Levulose*.—Reaction neutral.

*Maltose*.—Reaction neutral.

*Mannite*.—Reaction neutral.

*Agar*.—A white, nonspreading growth occurs.

*Serum*.—White, moderate, nonviscid, nonspreading growth occurs along the surface inoculated. No liquefaction takes place.

*Potato water*.—Reaction neutral.

*Potato*.—Gray, luxuriant, fleshy growth occurs.

*Milk*.—No change occurs.

*Litmus milk*.—No change occurs.

*Gelatin*.—A moderate growth is formed, accompanied by no liquefaction.

*Acid agar*.—Moderate growth takes place.

*Indol*.—Negative.

*Nitrates*.—Reduced to nitrites.

#### ***Saccharomyces roseus*.**

*Occurrence*.—Isolated from comb of healthy hive.

*Gelatin colonies*.—Superficial colonies are pink, convex, capitate, with lobate-lobulate margin; when magnified, the deep colonies are irregular, brownish-yellow, and finely granular.

*Morphology*.—This cell is oval, attaining about  $6.5\mu$  in length and  $3.5\mu$  in thickness. The cells stain uniformly.

*Motility*.—No motility occurs.

*Gram's stain*.—The cells are not decolorized by Gram's stain.

*Oxygen requirements*.—Aërobic.

*Bouillon*.—This medium remains clear, forming a pink, friable sediment. A pink band forms at the surface of the medium and adheres to the glass.

*Glucose*.—The closed arm remains clear. No gas is formed. Reaction acid.

*Lactose*.—Reaction neutral.

*Saccharose*.—Reaction neutral.

*Levulose*.—Reaction slightly acid.

*Maltose*.—Reaction slightly acid.

*Mannite*.—Reaction neutral.

*Potato water*.—Reaction acid.

*Glucose agar*.—Luxuriant, red growth forms on the surface.

*Serum*.—A pink, fleshy, nonspreading growth is formed, accompanied by no liquefaction.

*Potato*.—A thick, nonspreading, red growth occurs.

*Milk*.—No apparent change takes place. The milk coagulates on boiling.

*Litmus milk*.—Reaction alkaline.

*Gelatin*.—Moderate pink growth is formed, accompanied by no liquefaction.

*Acid agar*.—Slow growth occurs.

*Indol*.—Negative.

*Nitrates*.—Reduction to nitrites is positive.

#### ***Saccharomyces G.***

*Occurrence*.—Found in the dead larvæ of diseased adult bees.

*Morphology*.—They appear in hanging-drop preparation in large clusters,



stain uniformly with carbol-fuchsin and are oval, nearly spherical, attaining the length of  $4.5\mu$  and thickness of  $3.5\mu$ .

*Gram's stain.*—The cells are not decolorized by Gram's stain.

*Oxygen requirements.*—Aërobic.

*Bouillon.*—A slight, friable, white sediment is formed, with a clear medium above. Reaction slightly acid.

*Glucose.*—The medium in the closed arm remains practically clear and about one-fifth of the closed arm is filled with gas. Reaction acid.

*Lactose.*—Reaction neutral.

*Saccharose.*—Reaction neutral.

*Levulose.*—Reaction slightly acid.

*Maltose.*—Reaction slightly acid.

*Mannite.*—Reaction neutral.

*Potato water.*—Reaction acid.

*Agar.*—A moderate, white growth is formed.

*Serum.*—Very feeble growth occurs, accompanied by no liquefaction.

*Potato.*—A luxuriant, moist, white growth occurs.

*Milk.*—No appreciable change takes place.

*Litmus milk.*—No appreciable change takes place.

*Gelatin.*—A moderate, white growth occurs along needle tract and on the surface. No liquefaction results.

*Acid agar.*—A feeble white growth occurs.

*Indol.*—None could be demonstrated.

*Nitrates.*—No reduction to nitrites occurs.

*Glucose agar.*—A thick, white, fleshy growth occurs.





## SUMMARY TO PART I.

The results of the study of the bacteria found normally in the apiary may be briefly summarized as follows:

(1) The temperature of the hive approximates that of warm-blooded animals.

(2) Upon adult bees and upon the comb there occurs quite constantly a species of bacteria which we refer to in this paper as *Bacillus A*, and which, it is believed, is the organism that some workers have confused with *Bacillus alvei*, the cause of European foul brood (p. 33).

(3) There occurs very constantly in the pollen and intestine of adult bees a species here referred to as *Bacillus B*.

(4) From the combs *Bacterium cyaneus*, *Saccharomyces roseus*, and a *Micrococcus* referred to here as *Micrococcus C*, have been isolated and studied.

(5) Honey from a healthy hive is, as a rule, sterile.

(6) The normal larvæ are, as a rule, sterile.

(7) There is an anaërobe found quite constantly in the intestine of the healthy honey bee. It is referred to in this paper as *Bacterium D*.

(8) From the intestine there have been isolated and studied the following micro-organisms: *Bacillus cloacæ*, *Bacillus coli communis*, *Bacillus cholerae suis*, *Bacillus subgastricus*, *Bacterium mycoides*, *Pseudomonas fluorescens liquefaciens*, and two referred to as *Bacillus E*, and *Saccharomyces F*. Others less frequently present have been isolated, but not studied.

(9) In two samples of brood with unknown disease there was found a species of yeast plant here referred to as *Saccharomyces G*.

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## PART II.—THE DISEASES OF BEES.

The bee industry in this country, and other countries as well, is suffering large losses from various diseases among bees. Those which are most destructive attack the brood and weaken the colony by killing off large numbers of the young larvæ which would otherwise mature. There are other diseases which attack the adults and so decrease the strength of the colony in that way.

In order to combat a disease to the best advantage it is clear that its cause must be known, as well as the means by which the infection is transmitted and the environmental conditions which are favorable for the breaking out of an epidemic. The brood diseases among bees are on the increase. The custom of selling and shipping the honey, which is now carried on more extensively than formerly, the manner in which the products of the apiary are handled, and the absence of a general knowledge by the mass of bee keepers of the nature of the diseases are conditions which must be met before the spread of these diseases can be checked. When a colony is diseased, very little or no profit is realized from it; consequently the wealth and comfort of a very large number of people are greatly endangered by the existence of bee diseases. This suggests the importance, from an economic standpoint, of a thoro knowledge of these disorders.

### BRIEF HISTORY.

The attention of investigators has been attracted by these diseased conditions, not only from the economic interests attached thereto, but from the scientific point of view as well. The writings of Aristotle (12) contain an account of certain disorders which were then prevalent among bees; at that time it was thought that the blight of flowers bore a relation to bee diseases. In 1769 Schirach (13) gave the name foul brood to a diseased condition of the brood of bees; he attributed the cause to (a) unwholesome food, and (b) the placing of the larvæ with head inward in the cell. Leuckhart (14) thought the cause to be a fungus, related to the cause (*Panhistophyton ovatum*) of the disease of the silkworm. Muhlfield (15), in 1868, thought the trouble to be of two kinds—infectious and noninfectious—and that the cause of the infectious one is the larva of a parasitic fly (*Ichneumon apium mellificarium*) feeding upon the larvæ of the bee. In 1868 Preuss (16) exprest the view that the cause of



foul brood is a fermenting fungus belonging to the genus *Cryptococcus*. Geilen (17), in 1868, thought that when bees alight on the remains of animal bodies the putrefying matter thus carried with them may cause foul brood. The fermentation of bee bread was thought by Lambrecht (18) to be a sufficient cause of the disease; while Hallier (19) thought that various fungi could produce the disorder. On the contrary, Cornallia (20), in 1870, expressed the opinion that a fungus (*Cryptococcus alvearis*) is the specific cause of the trouble. Fischer (21), in 1871, supposed that a predisposing factor of foul brood is to be found in insufficient nourishment. In 1874 Cohn and Eidem received from Schonfeld samples of foul brood and, upon examination, they found spores and rods. In 1885 Cheshire and Cheyne (22) determined the cause and named the germ *Bacillus alvei*. Dickel (23) claimed that a number of different species might be the cause of foul brood. In 1900 Harrison (24) writes on foul brood and *Bacillus alvei*, its cause. Doctor Lambotte (25), in 1902, made some interesting studies concerning the relation of *Bacillus alvei* and *Bacillus mesentericus vulgaris*.

Since so many conflicting views have been held as to the cause of foul brood, one might conclude that the term "foul brood" has been applied incorrectly to a number of different disorders. In the light of more recent work this supposition is strengthened.

In June, 1902, the author, under the direction of Dr. Veranus A. Moore, began an investigation of bee diseases, especially as they existed in New York State. There were recognized at that time by bee inspectors of that State a number of distinct diseases which attacked the brood. Those which caused the greatest loss to the apiarists were known to the bee experts as "black brood," "foul brood," and "pickle brood." The results of the investigations of 1902 (26), 1903 (27), and 1904 (28) on these disorders, and on palsy or paralysis, are embodied in the following pages.

#### THE TERM "FOUL BROOD" AS HITHERTO APPLIED.

In the discussion of foul brood of bees it must be remembered that until recent years the name has been applied to what is now known to be two distinct diseases.

Schirach, in 1769, gave the name foul brood to a diseased condition in the brood of bees, but it is impossible to know to which of the two he referred. It may be that both diseases existed then as now and that he did not observe the fact that the two were different. We have reason to think that there are, at the present time in Europe, two distinct diseases to which the name foul brood is being applied. It is definitely known that such is the case in America.

It becomes necessary, then, to have two names to designate these

two diseased conditions in the brood of bees. For reasons given by Dr. E. F. Phillips, in the preface to this paper, it has been considered advisable to retain the name foul brood and to use a qualifying word to distinguish the two diseases. "European foul brood" and "American foul brood" are the names by which these two diseased conditions are to be designated.

In 1885 Cheyne (22) in England (Europe) found present in the decayed larvæ suffering from a diseased condition known as "foul brood" a new bacillus, which he named *Bacillus alvei* and to which he ascribed the cause of the disease. The diseased condition which contains *Bacillus alvei* is to be called "European foul brood," because this fact was first observed by an investigator working in Europe (England). In 1903 (27) the author observed that there was constantly present in the other diseased condition known as "foul brood" another bacillus which was new, and to which the name *Bacillus larvæ* is given. In view of the fact that *Bacillus larvæ* was constantly found to be present in the larvæ suffering from this disorder in the brood of bees, by investigations carried on in New York State (America) (27) (28), this diseased condition is to be called "American foul brood." From a scientific standpoint this choice of names for two distinct diseases might be easily criticized, but from the standpoint of the apiarist the selection of these names as the common ones for these two distinct disorders seemed almost necessary, or at least advisable.

#### EUROPEAN FOUL BROOD (FOUL BROOD OF CHEYNE).

The first scientific investigation of this disease bacteriologically was performed by Cheyne in 1885 (22). At this time he isolated a new bacillus from the dead larvæ. It was described by him and given the name *Bacillus alvei* (literally, hive bacillus). This afforded, then, a means for a positive diagnosis of this diseased condition.

#### Symptoms.

The symptoms of European foul brood, as given by Dr. E. F. Phillips in Circular No. 79, Bureau of Entomology, are as follows:

Adult bees in infected colonies are not very active, but do succeed in cleaning out some of the dried scales. This disease attacks larvæ earlier than does American foul brood, and a comparatively small percentage of the diseased brood is ever capped; the diseased larvæ which are capped over have sunken and perforated cappings. The larvæ when first attacked show a small yellow spot on the body near the head and move uneasily in the cell; when death occurs they turn yellow, then brown, and finally almost black. Decaying larvæ which have died of this disease do not usually stretch out in a long thread when a small stick is inserted and slowly removed; occasionally there is a very slight "ropiness," but this is never very marked. The thoroly dried larvæ form irregular scales which are not strongly adherent to the lower side wall of the



cell. There is very little odor from decaying larvæ which have died from this disease, and when an odor is noticeable it is not the "glue pot" odor of American foul brood, but more nearly resembles that of soured dead brood. This disease attacks drone and queen larvæ very soon after the colony is infected. It is, as a rule, much more infectious than American foul brood and spreads more rapidly. On the other hand, it sometimes happens that the disease will disappear of its own accord, a thing which the author never knew to occur in a genuine case of American foul brood. European foul brood is most destructive during the spring and early summer, often almost disappearing in late summer and autumn.

#### Confusion Regarding Foul Brood in America.

Prof. J. J. Mackenzie in 1882 made what seems to have been a short study of a bee disease as it appeared in Ontario, Canada, which was known to the apiarists of that Province as foul brood. He says very little of the character of the species of bacteria with which he was working, but he supposed that they were *Bacillus alvei* of Cheyne. The author has examined samples of brood from Ontario which have what, in the opinion of bee experts, is the most prevalent disease, and has not found *Bacillus alvei* present in any one. The bacteriological findings and the experience of bee-disease experts show that American foul brood is the prevalent disease in that Province. As the bee experts see the disease in the light of recent studies, there is no authentic report of which we are aware that European foul brood exists in Ontario. We can safely say, then, that *Bacillus alvei* can not be isolated from larvæ taken from the prevalent disease in the above-named Province. No difficulty is expressed on the part of Professor Mackenzie in the isolation of *Bacillus alvei* from any sample. The author is inclined to think, therefore, that this investigator was in error as to the identity of his culture, and therefore his conclusion can have little weight.

The foul brood of bees received some attention also from Prof. F. C. Harrison, of Ontario. In a paper of some length he gives a description of a species of bacteria which he identified as *Bacillus alvei*. The description which he gives and the accompanying photomicrographs (another plate which was given being after Cheyne and correct for *Bacillus alvei*) might easily be that of a member of a group represented by and described as *Bacillus* "A" in Part I of this paper. He also says that he has isolated *Bacillus alvei* from diseased larvæ from 13 States of the Union, ranging from New York to California and from Michigan to Florida. European foul brood has had a very limited geographical distribution, spreading only recently from New York to adjoining States. In Professor Harrison's work, too, there seems to have been no difficulty in isolating *Bacillus alvei* from diseased brood diagnosed by bee inspectors

as foul brood thruout the United States and Canada. In the experience of the author it has not been possible to obtain *Bacillus alvei* from diseased brood which the inspectors in most of the States and in Canada have been calling foul brood. For the above reasons the author believes that Harrison, too, has made a serious error in the identity of his culture and therefore was not working with *Bacillus alvei* at all. The author considers himself unfortunate in that he was unable to obtain a culture of *Bacillus alvei* for study and identification from Professor Harrison.

Dr. William R. Howard, of Fort Worth, Tex., also studied foul brood somewhat, and gave a description of *Bacillus alvei* as he found it. From his description and from the fact that he, too, worked with a diseased condition which does not contain *Bacillus alvei*, and express no difficulty in obtaining his cultures from any samples, the author believes that this investigator made an error in the identification of the culture with which he was working.

Some writers—Cowan, Bertrand, and others—have attempted the positive diagnosis of foul brood with the microscope alone from a preparation made direct from the dead larvæ. If the reader will remember that with the microscope alone it would be impossible to distinguish between *Bacillus larvæ* and *Bacillus alvei*, the verdict of these men can have no weight. As shown later in this paper under black brood (pp. 43-44), the Doctor Howard, of Fort Worth, Tex., referred to above, made an error in supposing that the European foul brood was a new disease and naming it "New York bee disease" or "black brood."

It is very unfortunate for the apiarist that these men should have fallen into error as to the identity of their culture with *Bacillus alvei*, as it has caused great confusion in the names of bee diseases. This confusion in the identity of cultures may be excused to a certain extent by the fact that European foul brood did not appear in this country, or at least did not attract much attention, until after Mackenzie, Harrison, and William R. Howard had done their work on foul brood.

#### The Present Investigation.

When the author's investigations were begun in 1902 there were two especially troublesome diseases in this country, which were then known to the bee experts as "black brood" and "foul brood."

The following summary and table shows the results of the examination of a number of samples of diseased brood from different apiaries, sent by the New York State bee inspectors during the summer of the year 1902:

Table showing the results of examinations of European foul brood. (The samples were called "black brood" by the apiarists at that time.)

Brood sent by—	Date.	Bacteriological findings.
W. D. Wright .....	June 4.....	<i>Bacillus alvei</i> .
W. D. Wright .....	June 12.....	<i>Bacillus alvei</i> .
N. D. West .....	June 12.....	<i>Bacillus alvei</i> .
N. D. West .....	June 12.....	<i>Bacillus alvei</i> .
N. D. West .....	June 12.....	<i>Bacillus alvei</i> .
N. D. West .....	June 12.....	<i>Bacillus alvei</i> .
N. D. West .....	June 12.....	<i>Bacillus alvei</i> .
N. D. West .....	June 12.....	<i>Bacillus alvei</i> .
N. D. West .....	June 12.....	<i>Bacillus alvei</i> .
N. D. West .....	Aug. 5.....	<i>Bacillus alvei</i> .
W. D. Wright .....	Oct. 8.....	<i>Bacillus alvei</i> .

It can be seen clearly from the above table that the diseased condition which the apiarists were calling "black brood" is really the disease "foul brood" of Cheshire and Cheyne, because of the constant presence of *Bacillus alvei*.

The work upon European foul brood was continued during the year 1903. The following table gives the results of the examination of specimens received during that year. The samples were taken from different apiaries.

Table giving a summary of the examination of specimens of European foul brood ("black brood").

Brood sent by—	Date.	Sources of brood in New York.	Bacteriological findings.
W. D. Wright.....	May 1	Columbia County .....	<i>Bacillus alvei</i> .
W. D. Wright.....	May 1	Albany County .....	<i>Bacillus alvei</i> .
N. D. West.....	June 25	Schoharie County .....	<i>Bacillus alvei</i> .
N. D. West.....	June 29	Schoharie County .....	<i>Bacillus alvei</i> .
N. D. West.....	June 29	Schoharie County .....	<i>Bacillus alvei</i> .
N. D. West.....	June 29	Schoharie County .....	<i>Bacillus alvei</i> .
N. D. West.....	June 29	Schoharie County .....	<i>Bacillus alvei</i> .
N. D. West.....	June 29	Schoharie County .....	<i>Bacillus alvei</i> .
N. D. West.....	July 6	Schoharie County .....	<i>Bacillus alvei</i> .
N. D. West.....	July 6	Schoharie County .....	<i>Bacillus alvei</i> .
N. D. West.....	July 6	Schoharie County .....	<i>Bacillus alvei</i> .
N. D. West.....	July 10	Montgomery County .....	<i>Bacillus alvei</i> .
N. D. West.....	July 10	Schoharie County .....	<i>Bacillus alvei</i> .
N. D. West.....	July 10	Schoharie County .....	<i>Bacillus alvei</i> .
N. D. West.....	July 10	Schoharie County .....	<i>Bacillus alvei</i> .
N. D. West.....	July 15	Schoharie County .....	<i>Bacillus alvei</i> .
N. D. West.....	July 15	Montgomery County .....	<i>Bacillus alvei</i> .
N. D. West.....	July 22	Schoharie County .....	<i>Bacillus alvei</i> .
N. D. West.....	July 22	Schoharie County .....	<i>Bacillus alvei</i> .
N. D. West.....	July 22	Schoharie County .....	<i>Bacillus alvei</i> .
N. D. West.....	July 30	Schoharie County .....	<i>Bacillus alvei</i> .
N. D. West.....	July 30	Schoharie County .....	<i>Bacillus alvei</i> .
N. D. West.....	July 30	Greene County .....	<i>Bacillus alvei</i> .
N. D. West.....	July 30	Albany County .....	<i>Bacillus alvei</i> .
N. D. West.....	July 30	Greene County .....	<i>Bacillus alvei</i> .
N. D. West.....	Aug. 20	Greene County .....	<i>Bacillus alvei</i> .
N. D. West.....	Aug. 20	Greene County .....	<i>Bacillus alvei</i> .

The above table shows that *Bacillus alvei* was present in each specimen of European foul brood received. Frequently pure cultures of this species were obtained from dead larvæ, but with it sometimes were associated other rod-shaped bacteria of different species.

In 1904 the work upon bee diseases was confined principally to the diagnosis of the diseased brood sent in and a further study of the organisms found. *Bacillus alvei* was found in a large number of



samples received from New York State and in some received from Pennsylvania.

**Bacillus alvei.**

*Occurrence.*—This bacillus was found in all samples of European foul brood examined.

*Morphology.*—The bacillus is a motile, rod-shaped organism, occurring singly and in pairs, and varying when taken from the surface of agar from  $1.2\mu$  to  $3.9\mu$  in length, and from  $0.5\mu$  to  $0.7\mu$  in width. Involution forms are sometimes present. Spores are produced and occupy an intermediate position in the organism. They are oval and vary from  $1.5\mu$  to  $2\mu$  in length and from  $0.7\mu$  to  $1\mu$  in breadth; they exhibit polar germination. The few flagella are arranged peritrichic.

*Oxygen requirements.*—This bacillus is a facultative anaërobe which grows at room temperature, but better at  $37^{\circ}\text{C}$ .

*Bouillon.*—The medium becomes uniformly clouded in 24 hours; later it shows a tendency to clear by a settling of the organisms. A somewhat viscid sediment is thus formed in the bottom of the tube. In older cultures a slightly gray band of growth adheres to the glass at the surface of the medium. The acidity is at first slightly increased, and a pellicle is sometimes formed.

*Glucose.*—The medium in both branches of the fermentation tube becomes uniformly clouded. Gas is not formed. Reaction acid.

*Lactose.*—The medium becomes uniformly clouded in both branches of the fermentation tube, but the cloudiness is not so marked as when glucose is used. The acidity is slightly increased, as shown by phenolphthalein. No gas is formed.

*Saccharose.*—The bouillon in this case also becomes clouded in both arms. A heavier growth is observed than when lactose is used, but less than when glucose is used. Acidity is slightly increased. Gas is not formed.

*Agar plates.*—Small, grayish, circular colonies form in 24 hours. When many are on the plate, they do not exceed 2 millimeters in diameter. Under low magnification they appear granular, with no definite margin. When fewer colonies are on the plate, the granular center of the colony is surrounded by numerous smaller but similar growths. The organism has a tendency to grow into the medium rather than upon the surface. Sometimes, however, when there are but a few colonies on the plate a thin, transparent growth spreads rapidly over the surface. Later it takes on a brown tint.

*Agar slant.*—A gray layer spreads over the surface in 24 hours, which later takes on a slightly brown color. A strong, slightly viscid growth occurs in the condensation water.

*Acid agar.*—Growth takes place with the reactions varying from neutral to +3.5 to phenolphthalein.

*Serum.*—A slightly raised growth which is confined quite closely to the line of inoculation appears on the surface of solidified serum.

*Potato.*—On this medium the bacillus grows rather slowly at first, but after 3 or 4 days a milky growth is observed, which increases until a luxuriant growth is formed, which varies from a lemon-yellow to a gray color, and which later becomes tinted with brown.

*Milk.*—Acidity is increased after inoculation. Coagulation usually takes place after the third day.

*Litmus milk.*—Much of the blue color is discharged, leaving the coagulated milk of a light brown.

*Gelatin colonies.*—Gelatin is a medium in which it develops slowly. The colony becomes very irregular in outline, owing to thread-like outgrowths which take place in curves from its border. Growth is better when 5 per cent glycerin is added. From the small, white, spherical colonies which form along the line of puncture gray, thread-like growths shoot out thru the medium. In about 2 months the gelatin is changed to a thick liquid, holding gray flocculent masses of organisms which gradually settle, forming a strong, slightly viscid sediment.

*Indol.*—In old cultures a decided indol reaction is obtained.

*Power to resist disinfectants.*—Preliminary observations give the following results: The spore form resists drying for a considerable time. Spores which have been drying for 1 year germinate promptly when introduced into bouillon. The vegetative form: One per cent carbolic acid kills in 10 minutes; 3 per cent carbolic acid kills in 2 minutes; mercuric chlorid solution, 1 to 1,000, kills in 1 minute; mercuric chlorid solution, 1 to 2,000, kills in 2 minutes.

*Spore form.*—Mercuric chlorid, 1 to 1,000, kills in 30 minutes.

*Pathogenesis in vertebrates.*—Inoculations into guinea pigs and frogs have not proven this organism to be pathogenic to these animals.

### Inoculation Experiments.

That part of the investigation which involves the producing of the disease experimentally by inoculating with pure cultures of the organism is usually the most difficult one. Very rarely indeed is one able to produce the disease with symptoms closely simulating those found in nature. The experimental production of a disease involves many variable factors, such as attenuation of the organism, methods of inoculation, resistance of the host, and the immediate environment.

On August 4, 1902, we inoculated a hive containing nothing but healthy brood, free from bacteria, by feeding with sirup (sugar and water in equal parts) to which was added the growth from the surface of the plate cultures containing spores and bouillon cultures of *Bacillus alvei*. Similar feedings were given to these bees from one to three times a week until September 28, but symptoms of foul brood did not develop. On August 6 cultures were made from a few of the hive larvæ. They were found to contain the bacilli.

Inoculation experiments were again made in 1903. Because of a failure to produce a diseased condition with cultures of *Bacillus alvei* in the experiment of 1902, the variable factors above mentioned were carefully considered in the experiment of this year. The inoculations were made when climatic conditions were such as seemed to favor the ravages of the disease in the apiaries; namely, low temperature, dampness, and cloudiness. A colony of black bees was used, as they were almost universally considered more susceptible. Cultures of *Bacillus alvei* were freshly isolated from foul-brood specimens and kept in stock on bee-larvæ agar (described under American foul brood, pp. 41–42). All cultures were incubated at 34° C., which temperature is observed to be slightly below that of the hive. The spore form of *Bacillus alvei* was used.

Inoculations were made in different ways. A diseased condition



appeared in the hive when the following method was used: The agar from plates on which the culture was grown was finely crushed and mixed with sterile sirup. A jelly glass, in the lid of which holes had been punctured, was filled and inverted on strips of wood inside the hive. In this way the bees take up the culture with the sirup as rapidly as it flows out of the glasses. A colony having brood free from *Bacillus alvei* was fed in the above manner on August 8, with repeated feedings on the 9th, 10th, 12th, 13th, 15th, and 17th. On the 12th *Bacillus alvei* was found in the living larvæ and on the 17th many larvæ were dead under cappings and some were dead which were not capped; all were soft and of a dull color. Many of the capped cells containing dead larvæ had their capping freshly punctured. *Bacillus alvei* was usually obtained from these larvæ in pure cultures. In no cell examined where the cell capping was punctured did we find gas-producing organisms; this fact would suggest the conclusion that these punctures which are found in the capping in foul brood are made by the bees and not by gas-producing organisms. During this series of inoculations the days were quite cool and sometimes cloudy and damp. On the 20th of August the temperature was much higher, the bees were more active, and much of the dead brood had been cleaned out by the bees. On the 22d no dead brood was noticed by casually looking over the brood nest. On the 24th of the same month a careful search was made by uncapping all the cells of one brood frame, and 12 decaying larvæ of a brown color were found. At this time the larvæ were not viscid. All the remaining dead brood had evidently been cleaned out by the bees. A condition similar to this, where only a few scattered about in the brood nests contain dead larvæ, occurs sometimes in affected apiaries. Two other colonies which were near by but not inoculated gave no signs of disease.

Mr. N. D. West reports that the climatic conditions seem to have something to do with the extent of the ravages of European foul brood, since the disease is much more destructive in cool, damp weather. This seems to be a very plausible idea. The larvæ at such times may receive more infected food than when fresh is being rapidly gathered; the resistance of the body of the larvæ to the growth of *Bacillus alvei* is at such times much lessened; and the adult bees being less active, the dead larvæ are not cleaned out of the combs so rapidly. The results of the experimental work seem to confirm this theory.

#### Distribution of *Bacillus alvei* in Infected Hives.

In order to combat this disease it is well to know where these pathogenic bacteria may be found. The following is a summary of the results of the investigation along this line:



1. The greatest number of infecting germs are found in the bodies of dead larvæ.

2. The pollen stored in the cells of the foul-brood combs contains many of these infecting organisms.

3. The honey stored in brood combs infected with this disease has been found to contain *Bacillus alvei* in small numbers.

4. The surface of the combs, frames, and hives may be contaminated.

5. The wings, legs, head, thorax, abdomen, and intestinal contents of adult bees are found to be contaminated with *Bacillus alvei*.

6. Cheshire (29), Mackenzie (30), and others have found *Bacillus alvei* in the ovary of the queen. This has suggested a means of infection. From a bacteriological examination of queens from three badly infected hives we were able to isolate *Bacillus alvei* in small numbers in two cases. Since a very large number of this species of bacteria may be found in the intestinal tract and upon all parts of the body, it is very probable that such findings are the results of contamination in making cultures and have no special significance.

#### Experiments with Formaldehyde Gas.

Within the last few years several articles have appeared in the bee journals entertaining great hopes that a cure for foul brood has been found in the use of formaldehyde gas. The methods described for its use have been tested by the apiarists and bee experts in New York State, with the result that the disease sometimes breaks out anew in colonies so treated.

In order to test the value of formaldehyde gas as a disinfectant when used in foul-brood combs a number of experiments were made in the laboratory. A common frame hive was first used, in which were placed specimens of foul brood. The hive was charged with gas by heating formalin in a closed vessel which was in communication with the hive; 15 c. c. was used each time and evaporated to dryness. The charging of the hive with gas was repeated in this way at the end of 2, 4, 6, and 20 hours. Before each charging and at the end of 24 hours after the first application of gas, cultures were made. Of all the tubes inoculated 90 per cent showed *Bacillus alvei* to be present. There was no decrease in the number of tubes in which *Bacillus alvei* appeared following the several applications of formaldehyde gas.

The examination of specimens of foul brood which had been treated with the gas by an apiarist gave the following results:

Thirty tubes which were inoculated from larvæ, capped and uncapped, showed the presence of *Bacillus alvei* in 21.

Thirty tubes which were inoculated with pollen in cells gave *Bacillus alvei* in 28.

Four series of agar plates showed apparently no diminution in the number of bacteria present.

Further experiments were made by using Novy's anaërobic jar (a very tight chamber) as a chamber in which to put the diseased brood combs and cultures. This vessel will retain the gas much more perfectly than the devices made for practical use in the apiary. Treatment of brood in this jar by recharging with the gas resulted usually in complete disinfection after 2 days. Agar plates containing spores and cheese cloth on which cultures were spread and dried were disinfected after a short length of time by the application of formaldehyde gas.

From the experiments made the conclusion can be drawn that formaldehyde gas is a good disinfectant, but that it penetrates very slowly and that 24 hours' application of the gas to the combs, as usually applied, is not sufficient to kill all the spores in the decayed larvæ (27).

#### AMERICAN FOUL BROOD.

The diseased condition which we shall call American foul brood and the micro-organism found constantly present in the diseased and dead larvæ, which we shall call *Bacillus larvæ*, were, for convenience, referred to, respectively, as "X Brood" and *Bacillus* "X" in a former report (27). This disease has been called "foul brood" by many bee keepers in this country and in other countries as well. It is the diseased condition with which Mackenzie, Harrison, and William R. Howard were working largely, if not altogether, in their investigations of foul brood. The disorder is, as a rule, dreaded less than European foul brood by the apiarist, yet in the aggregate the bee industry suffers enormous losses from the trouble. The general character of the diseased brood is so much like that of foul brood that the two may be easily confused by those unfamiliar with the variety of appearances which one finds in each disease and a few characters which are differential. Therefore it is not strange that the mistaken diagnosis should be made from the symptoms manifested by these two diseases. When, however, European foul brood and American foul brood are subjected to a bacteriological examination, the diagnosis is easy. Experts when comparing specimens of the two diseased conditions are able to see a difference in the gross appearance.

#### Symptoms.

The symptoms are given by Dr. E. F. Phillips in Circular No. 79, Bureau of Entomology, as follows:

The adult bees of an infected colony are usually rather inactive and do little toward cleaning out infected material. When the larvæ are first affected they turn to a light chocolate color, and in the advanced stages of decay they become

darker, resembling roasted coffee in color. Usually the larvæ are attacked at about the time of capping, and most of the cells containing infected larvæ are capped. As decay proceeds these cappings become sunken and perforated, and, as the healthy brood emerges, the comb shows the scattered cells containing larvæ which have died of disease still capped. The most noticeable characteristic of this infection is the fact that when a small stick is inserted in a larva which has died of the disease, and slowly removed, the broken-down tissues adhere to it and will often stretch out for several inches before breaking. When the larva dries it forms a tightly adhering scale of very dark brown color, which can best be observed when the comb is held so that a bright light strikes the lower side wall. Decaying larvæ which have died of this disease have a very characteristic odor, which resembles a poor quality of glue. This disease seldom attacks drone or queen larvæ. It appears to be much more virulent in the western part of the United States than in the East.

A microscopic preparation from the diseased, but not dead larvæ, or from larvæ recently dead, at first shows a few comparatively long slender rods; later these increase rapidly in number, and spores also are seen. In the later stages of decay in the ropy mass and the dried scales spores only are found; these occur in very large numbers. When this investigation was begun, in 1902, it was observed (26) that in the dried dead larvæ there are very large numbers of spores, but these, when inoculated into the media commonly used in the laboratory, fail to grow. The cultures were sterile, except for an occasional contamination.

#### The Present Investigation.

The following samples from different sources were examined in 1902:

*Results of examination of specimens of American foul brood diagnosed by the experts at that time simply as "foul brood."*

Brood sent by—	Date.	Source.	Bacteriological findings.
Charles Stewart .....	June 12.....	New York.....	No growth.
W. D. Wright.....	Sept. 19.....	Wisconsin.....	2 unidentified bacilli.
W. D. Wright.....	Oct. 19.....	Canada.....	No growth.
W. D. Wright.....	Nov. 11.....	Wisconsin.....	No growth; 4 samples.

Inasmuch as *Bacillus alvei* was absent, it is evident that this condition is not European foul brood (26).

In 1903 the investigations were continued. Several media were devised in which it was hoped that it would be possible to obtain a germination of the spores which were observed the year before and which failed to grow on our ordinary media. The one which proved successful was prepared as follows: Larvæ are picked from the brood combs of a number of frames of healthy brood and a bouillon (bee-larvæ bouillon) is made from them following the same directions as when bouillon is made from meat. Our first growth from these



spores was secured in an agar (bee-larvæ agar) made from this special bouillon when Liborius's method for cultivating anaërobes was used.

The technique for making cultures successfully from the diseased material is not difficult if the following method is used: Place a loopful of the decayed tissue of the larvæ into a tube of bouillon; heat to 65° C. for 10 minutes to kill any vegetative forms which might be present; incubate for 12 hours, and heat again to 65° C. for 10 minutes. This is usually sufficient, but it may be necessary to repeat the same process. Liquefied bee-larvæ agar in a test tube is then inoculated and incubated. The successive heating will destroy the vegetative stage of any spore-producing species which is common about the apiary, *e. g.*, members of the group represented by *Bacillus A*, as described on pp. 13-14 of this paper. Agar slant and bouillon, when inoculated from this source, remain sterile; but when bee-larvæ agar is used a slow but abundant growth takes place. Under certain conditions the growth appears very near or at the surface when cultures are made in the above manner. A surface growth can be obtained after a few generations by reinoculating slant agar of this same medium.

The above method was used successfully in diagnosing the following samples from different apiaries:

*Results of examination of specimens of American foul brood, formerly called simply "foul brood."*

Brood sent by—	Date.	Source.	Bacteriological findings.
W. D. Wright.....	Oct. 19, 1902	Canada.....	<i>Bacillus larvæ.</i>
W. D. Wright.....	Nov. 11, 1902	Wisconsin.....	<i>Bacillus larvæ.</i>
W. D. Wright.....	Nov. 11, 1902	Wisconsin.....	<i>Bacillus larvæ.</i>
C. H. W. Weber.....	July 24, 1903	Ohio.....	<i>Bacillus larvæ.</i>
N. D. West.....	Aug. 3, 1903	Broome County, N. Y.....	<i>Bacillus larvæ.</i>
N. D. West.....	Aug. 3, 1903	Broome County, N. Y.....	<i>Bacillus larvæ.</i>
N. D. West.....	Aug. 3, 1903	Chenango County, N. Y.....	<i>Bacillus larvæ.</i>

The results of these examinations show that *Bacillus larvæ* was present in all the specimens examined, which suggests that it very probably figures as an etiological factor in this disease. Other bacteria of different species are occasionally found associated with this bacillus.

#### **Bacillus larvæ.**

*Occurrence.*—Constantly present in diseased brood from colonies affected with American foul brood.

*Gelatin.*—There is no growth.

*Morphology.*—It is a slender rod, having a tendency to form in chains. This is especially true when grown in bee-larvæ bouillon.

*Motility.*—The bacillus is rather sluggishly motile.

*Spores.*—Spore formation takes place. This can be observed best in the different stages of the disease and decay of the larvæ.

*Oxygen requirements.*—When Liborius's method is used, the best growth usually appears near to but not on the surface. After a few generations a surface growth may be obtained.

*Bouillon*.—There is no growth.

*Glucose bouillon*.—There is no growth.

*Lactose*.—There is no growth.

*Saccharose*.—There is no growth.

*Agar plate*.—There is no growth.

*Bee-larvæ agar*.—The inoculations must be made with the medium liquefied. The growth takes place near to but rarely on the surface. Cultures must pass thru a few generations before a satisfactory surface growth can be secured.

*Bee-larvæ agar slant*.—On the surface of this medium a thin, gray, nonviscid growth takes place.

*Glucose agar*.—Slight growth has been observed in the medium. No gas is produced.

*Potato*.—There is no growth.

*Milk*.—There is no growth.

*Litmus milk*.—There is no growth.

*Fermentation*.—In bee-larvæ bouillon no gas is produced.

*Indol*.—There is no growth in sugar-free bouillon.

### THE SO-CALLED "PICKLE BROOD."

The name "pickle brood" was given by Dr. William R. Howard, of Fort Worth, Tex., to a disorder found in the brood of bees. He stated that the cause of the disease was a specific fungus which he called *Aspergillus pollinis*. His results have not been confirmed by other investigators.

The bee keepers are sustaining a loss from a diseased condition in their apiaries which they are diagnosing as "pickle brood." The larvæ usually die late in the larval stage. Most of them are found on end in the cell, the head frequently blackened and the body of a watery, granular consistency.

The following table gives a summary of the results of an examination of specimens received labeled "pickle brood:"

*Results of examination of specimens of so-called "pickle brood."*

Brood sent by—	Date.	Bacteriological findings.
W. D. Wright.....	June 17, 1902.....	Two unidentified micrococci.
W. D. Wright.....	July 31, 1902.....	No growth.
W. D. Wright.....	Aug. 4, 1902.....	No growth.
M. Stevens.....	Aug. 20, 1902.....	Unidentified bacilli.
W. D. Wright.....	Sept. 2, 1902.....	Unidentified bacilli.
W. D. Wright.....	June 24, 1903.....	Unidentified bacilli and yeast.
N. D. West.....	Aug. 5, 1903.....	No growth.
M. Stevens.....	Aug. 20, 1903.....	No growth.

The results of the examinations show that *Aspergillus pollinis* was not found. Further investigations must be made before any conclusion can be drawn as to the real cause of this trouble.

### THE SO-CALLED "BLACK BROOD."

In 1890 some specimens of diseased brood were sent from New York State to Dr. William R. Howard, of Fort Worth, Tex., and unfortunately, after a short and inadequate study of the disease, he

reported it to be a new disease and called it "New York bee disease" or "black brood." He described as its cause a species of bacteria which he called *Bacillus millii* (31).

In our investigations of this diseased condition, which have covered five years, we have not found an organism corresponding to *Bacillus millii* in any of the specimens that we have received; but we have found *Bacillus alvei*, the supposed cause of foul brood, to be present constantly in samples of brood which the bee experts of New York State say are samples of the same diseased condition as that received by Howard.

From this we conclude that the diseased brood that has received the name of "New York bee disease" or "black brood" is really genuine European foul brood.

#### PALSY OR PARALYSIS.

The disease known to the apiarists as palsy or paralysis attacks the adult bees. The name is suggestive of the symptoms manifested by the diseased bees. A number of bees affected were received from Messrs. W. D. Wright and Charles Stewart, taken from apiaries in New York State. In 1903 bacteriological examinations were made of a number of bees so affected. Several species of bacteria were isolated and some experimental inoculations made, but no conclusions could be drawn from the results obtained as to the cause of the disorder.

From a study of the normal flora of the bee it was soon found that we had here quite a number of species of bacteria present. This fact stimulated a study of the normal flora, the results of which are recorded in Part I. From this point the work can be carried on with the hope that, if the disease has a bacterium as an etiological factor, it may be found. It is believed by some bee keepers that *Bacillus gaytoni* of Cheshire is the cause of paralysis, but this is not claimed by Cheshire, and the belief is not grounded on bacteriological findings.

#### SUMMARY TO PART II.

Following is a brief summary of the results of the present investigation of bee diseases:

(1) There are a number of diseased conditions which affect the apiary.

(2) The disease which seems to cause the most rapid loss to the apiarist is European foul brood, in which is found *Bacillus alvei*—first isolated, studied, and named by Cheshire and Cheyne in 1885.

(3) The distribution of *Bacillus alvei* in the infected hive is as follows:

(a) The greatest number of infecting germs are found in the bodies of dead larvæ.

(b) The pollen stored in the cells of the foul-brood combs contains many of these infecting organisms.



(c) The honey stored in brood combs infected with this disease has been found to contain a few bacilli of this species.

(d) The surface of combs, frames, and hives may be contaminated.

(e) The wings, head, legs, thorax, abdomen, and intestinal contents of adult bees were found to be contaminated with *Bacillus alvei*.

(f) *Bacillus alvei* may appear in cultures made from the ovary of queens from European foul-brood colonies, but the presence of this species suggests contamination from the body of the queen while the cultures are being made and has no special significance.

(4) The disease which seems to be most widespread in the United States we have called American foul brood, and the organism which has been found constantly present in the disease we have called *Bacillus larvæ*. This disorder was thought by many in this country and other countries as well to be the foul brood described by Cheshire and Cheyne, but such is not the case.

(5) From the nature of American foul brood it is thought that the organism has a similar distribution to that of *Bacillus alvei*.

(6) It appears that European foul brood was erroneously called "New York bee disease" or "black brood" by Dr. Wm. R. Howard in 1900.

(7) There is a diseased condition affecting the brood of bees which is being called by the bee keepers "pickle brood." No conclusion can be drawn from the investigation so far as to the cause of the disease.

(8) *Aspergillus pollinis*, ascribed by Dr. William R. Howard as the cause of pickle brood, has not been found in this investigation and is not believed by the author to have any etiological relation to the so-called "pickle brood."

(9) Palsy or paralysis is a diseased condition of the adult bees. No conclusion can yet be drawn as to its cause.

(10) Formaldehyde gas as ordinarily used in the apiaries is insufficient to insure complete disinfection.

#### CONCLUSIONS.

In a paragraph the author wishes, if possible, to present the status of the bee diseases in this country. It should be remembered, firstly, that "black brood" can now be dropt from our vocabulary, and probably does not exist; secondly, that the term "foul brood" was being applied to two distinct diseases. One of these diseases we now refer to as European foul brood, because it first received a scientific study from a European investigator. We refer to the other disease as American foul brood, because it was first studied scientifically in America. There is one more disorder in the brood of bees which has attracted considerable attention—the so-called "pickle brood." There are, then, these three principal diseases: European foul brood, American foul brood, and the so-called "pickle brood."

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